

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|  |           |   |
|--|-----------|---|
| <b>(51) International Patent Classification <sup>5</sup> :</b><br>C12N 15/18, C12P 21/02<br>C07K 13/00, A61K 37/36<br>A23K 1/165, C12N 1/21<br>// (C12N 1/21, C12R 1/19)   | <b>A1</b> | <b>(11) International Publication Number:</b> <b>WO 90/08823</b><br><br><b>(43) International Publication Date:</b> 9 August 1990 (09.08.90)  |
| <b>(21) International Application Number:</b> PCT/US89/05445<br><b>(22) International Filing Date:</b> 11 December 1989 (11.12.89)<br><br><b>(30) Priority data:</b><br>304,733 31 January 1989 (31.01.89) US<br><br><b>(60) Parent Application or Grant</b><br><b>(63) Related by Continuation</b><br>US 304,733 (CON)<br>Filed on 31 January 1989 (31.01.89)<br><br><b>(71) Applicant (for all designated States except US):</b> THE UPJOHN COMPANY [US/US]; 301 Henrietta Street, Kalamazoo, MI 49001 (US). |           | <b>(72) Inventors; and</b><br><b>(75) Inventors/Applicants (for US only) :</b> PARCELLS, Alan, J. [US/US]; 1310 Sussex Street, Portage, MI 49002 (US). MOTT, John, E. [US/US]; 4720 East C Avenue, Kalamazoo, MI 49004 (US). TOMICH, Che-Shen, C. [US/US]; 3703 Blackberry Lane, Kalamazoo, MI 49008 (US).<br><br><b>(74) Agent:</b> KOIVUNIEMI, Paul, K.; Patent Law Department, The Upjohn Company, Kalamazoo, MI 49001 (US).<br><br><b>(81) Designated States:</b> AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), DK, ES (European patent), FI, FR (European patent), GB (European patent), HU, IT (European patent), JP, KR, LU (European patent), NL (European patent), NO, SE (European patent), SU, US.<br><br><b>Published</b><br><i>With international search report.</i> |
| <b>(54) Title:</b> SOMATOTROPIN ANALOGS<br><br><b>(57) Abstract</b><br><br>This invention provides mammalian somatotropin analogs, and in particular, bovine somatotropin analogs in which at least one of the cysteine amino acids at positions 54, 164, 181 or 189 has been replaced with serine. Also provided are methods for enhancing growth and lactation using such analogs.   |           |   |

***FOR THE PURPOSES OF INFORMATION ONLY***

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                              |    |  |    |                          |
|----|------------------------------|----|--|----|--------------------------|
| AT | Austria                      | ES | Spain                                    | MG | Madagascar               |
| AU | Australia                    | FI | Finland                                  | ML | Mali                     |
| BB | Barbados                     | FR | France                                   | MR | Mauritania               |
| BE | Belgium                      | GA | Gabon                                    | MW | Malawi                   |
| BF | Burkina Faso                 | GB | United Kingdom                           | NL | Netherlands              |
| BG | Bulgaria                     | HU | Hungary                                  | NO | Norway                   |
| BJ | Benin                        | IT | Italy                                    | RO | Romania                  |
| BR | Brazil                       | JP | Japan                                    | SD | Sudan                    |
| CA | Canada                       | KP | Democratic People's Republic<br>of Korea | SE | Sweden                   |
| CF | Central African Republic     | KR | Republic of Korea                        | SN | Senegal                  |
| CG | Congo                        | LJ | Liechtenstein                            | SU | Soviet Union             |
| CH | Switzerland                  | LK | Sri Lanka                                | TD | Chad                     |
| CM | Cameroon                     | LU | Luxembourg                               | TG | Togo                     |
| DE | Germany, Federal Republic of | MC | Monaco                                   | US | United States of America |
| DK | Denmark                      |    |  |    |                          |

-1-

## SOMATOTROPIN ANALOGS

## FIELD OF INVENTION

This invention relates to analogs of animal somatotropins. More specifically, the invention relates to bovine somatotropin analogs.

## 5 BACKGROUND OF THE INVENTION

Somatotropins were originally discovered in pituitary gland extracts from various animal species. In general, somatotropins are conserved molecules and similarities in amino acid sequences and structure are found between different species of animals. Bovine  
10 somatotropin (or growth hormone) has been well studied. The literature was reviewed by Paladini, A.C. et al., CRC Crit. Rev. Biochem. 15:25-56 (1983).

Somatotropins, including bovine somatotropin, are globular proteins comprising a single chain of approximately 200 amino acids,  
15 having two intramolecular disulfide bonds. Specifically, the most common form of natural bovine somatotropin (bSt) has a single 190-191 amino acid chain, a globular structure with two intramolecular disulfide bonds and a molecular weight of about 22,000 daltons.

Natural bSt extracted from pituitary glands is, however, a  
20 heterogeneous mixture of proteins. At least six major forms of the protein have been described. The longest form has 191 amino acid residues and an ala-phe amino terminus. The second form has 190 amino acid residues and a phe amino terminus. The third form has 187 amino acid residues and a met amino terminus. The remaining three  
25 forms of bSt substitute valine for leucine at position 127. In addition to this heterogeneity, undefined heterogeneity of bSt has also been described (Hart, I.C. et al., Biochem. J. 218:573-581 (1984); Wallace, M. and Dickson, H.B.F., Biochem. J. 100:593-600 (1965)). Undefined electrophoretic heterogeneity is seen when native  
30 extracts are fractionated by anion exchange chromatography. It has been shown that the defined forms have different relative potency in bioassays. Also, it has been shown that other undefined species of bSt when fractionated on ion exchange columns demonstrate varying degrees of bioactivity in rat growth models (Hart, et al. and  
35 Wallace and Dickson, supra).

It is not known whether undefined heterogeneity is due to genetic variability, to in vivo post-translational modification, to differences in phosphorylation (Liberti, J.P. et al., Biochem. and

-2-

Biophys. Res. Comm. 128:713-720, 1985) or to artifacts of isolation.

bSt produced by recombinant microorganisms, or extracted from pituitary gland tissue, is important commercially. It increases lactation in dairy cattle and increases size and meat production in beef cattle. It is estimated that upwards to 20 mg per animal per day will be needed to effect commercially acceptable improvements. Such a dosage will require efficient methods of administration. Improvements in the potency of bSt such as described in this invention will be of benefit because of resulting reductions in the amount of drug administered to each animal per day.

Furthermore, one of the major problems in recovering recombinantly-produced proteins is the proper formation of the disulfide linkages. bSt has two such disulfide bridges in the native state. bSt contains four cysteine residues located at amino acid positions 53, 164, 181 and 189 of mature bSt. The first two form a disulfide bond which generates the so-called large loop while the latter two form the small loop of bSt. The recombinant bovine somatotropin (rbSt) protein produced in *E. coli* is in a reduced form and requires in the isolation protocol an oxidation step to form the disulfide bridges. It has been observed that reducible dimeric and polymeric forms of the protein are formed. Dimer has been shown to have reduced biopotency in the rat bioassay and reduced milk production in dairy cows. The dimeric rbSt may also be antigenic. The present invention overcomes these problems by eliminating the disulfide bridges, in particular, between cysteines 181 and 189.

#### INFORMATION DISCLOSURE

Analogues of bSt are known (see, for example, European patent applications 75,444 and 103,395 and Nucleic Acid Res. 10(20):6487 (1982)).

G. Winter and A.R. Fersht, TIBS, 2, p. 115 (1984) review the alteration of enzyme activity by changing amino acid composition at key sequence locations. They refer to the change of a cysteine to a serine residue in an enzyme with retention of enzyme activity.

L. Graf, et al, Int. J. Peptide Prot. Res., 7, pp. 467-73 (1975) demonstrated that the disulfide linkage between cysteine-181 and cysteine-189 is readily cleaved under mild conditions, i.e., in the absence of a denaturing agent. They also reported retention of growth-promoting activity by the reduced bSt as measured in a rat

-3-

tibia test. This activity was dependent upon blocking the resulting thiol groups with an uncharged alkylating reagent such as iodoacetamide. They demonstrated that if alkylation was done with iodoacetamide rather than iodoacetic acid there was no significant loss of growth-promoting activity.

M. Schleyer, et al, Hoppe-Seyler's Z. Physiol. Chem., 364, p. 291 (1983) described studies in which the disulfide bridges of porcine growth hormone were cleaved by a variety of techniques with retention of biological activity.

M. Schleyer, et al, Hoppe-Seyler's Z. Physiol. Chem., 363, p. 1111 (1982) refer to cleavage of the disulfide bridges of human growth hormone without completely abolishing biological activity.

A. Wang, et al, Science, 224, p. 1431 (1984), U.S. patent 4,518,584 and U.K. patent application 2,130,219A refer to the replacement of cysteine residues in interleukin-2 by serine and the effect of the changes on biological activity. At least two of the three cysteines appeared to be required for full biological activity.

P. Mantsala and H. Zalkin, J. Biol. Chem., 259, p. 14230 (1984) refer to the effect of replacing a cysteine in the enzyme glutamine amidotransferase by a phenylalanine residue. This change abolished the ability of the enzyme to activate the amide from glutamine.

U.S. patent 3,264,186 describes the limited cleavage of bovine growth hormone at methionine to produce an active protein.

T. Tokunaga, et al, Eur. J. Biochem. 153:445-449 (1985) refer to changing the cysteine at position 165 to alanine in human growth hormone to remove the disulfide bond between cys-53 and cys-165. They found the ala-165 molecule has full biological activity as examined by the body weight increase of hypophysectomized rats.

#### SUMMARY OF THE INVENTION

This invention relates to the enhancement of bioactivity, processability, and product uniformity of animal somatotropins having a cysteine to serine change in amino acids in at least one of positions 53, 164, 181 and 189 as exemplified in bSt (cys → ser) and similar changes in somatotropins from other species including porcine and ovine.

More specifically, the invention relates to mammalian somatotropins in which at least one of the cysteine residues corresponding to residues 53, 164, 181 and 189 is replaced with serine.

-4-

More specifically disclosed are mammalian somatotropins in which both cysteine residues corresponding to residues 181 and 189 are replaced with serine.

5 Even more specifically, are those bSt-analogs wherein the cysteines located at amino acid residues 181 and 189 are converted to serine (cys → ser bSt).

Specifically included are animal somatotropins in which the cysteine residue corresponding to residue 181 is replaced with serine or in which the cysteine residue corresponding to residue 189 is replaced with serine. More specifically, the animals are mammals,  
10 even more specifically bovines.

Also specifically included are animal somatotropins wherein at least one cysteine residue at position 53 or 164 is replaced with serine, more specifically, wherein the cysteine residue 53 is replaced with serine, and more specifically wherein the cysteine  
15 residue 164 is replaced with serine. More specifically, the animals are mammals, even more specifically bovines.

Also provided are methods for enhancing the growth of animals that comprises treatment of the animals with an effective amount of one of the disclosed animal somatotropins, specifically wherein the  
20 animal is a mammal, more specifically, wherein the mammal is a bovine.

Also provided are methods for increasing milk production in a female ruminant comprising the administration of an effective amount of a animal somatotropin of this invention, more specifically,  
25 wherein the ruminant is a dairy cow and wherein the somatotropin is bSt.

Also provided are vectors comprising DNA coding for an animal somatotropin having at least one of the cysteine residues corresponding to residues 53, 164, 181 and 189 changed to serine, specifically  
30 which are capable of directing the expression of the somatotropin-like protein.

Also provided are microorganisms hosting said vectors, specifically from the bacterial genus, Escherichia.

35 Both chemical and genetic modifications of these amino acid residues are embraced by this invention.

The preferred genetic modifications rely upon single site specific mutation methods for insertion of serine residues in

-5-

replacement of the naturally occurring cysteines.

#### DETAILED DESCRIPTION

Due to the molecular heterogeneity of somatotropins, the position numbers of amino acid residues of the various somatotropins may differ. The term "native mammalian somatotropin" includes these naturally occurring species. Chart 1 illustrates one species of bSt and the amino acid numbering that corresponds to the modification sites of this invention. The numbering for other somatotropins may differ. However, using the cysteines numbered 53, 164, 181 and 189 of the bSt in Chart 1, those of ordinary skill in the art can readily locate corresponding amino acid sequences in other native animal somatotropins, or their analogs, to achieve the desired enhanced bioactivity, processability and product uniformity.

The phrase "closest-related native somatotropin" refers to the naturally-occurring form of animal somatotropin which when compared to a specific somatotropin analog of the instant invention is more closely identical in amino acid sequence than any other naturally-occurring form of animal somatotropin. For example, the bSt of Chart 1 is the "closest-related native somatotropin" to an analog wherein the cysteine at position 53 is replaced with serine. The phrase "somatotropin-like protein" refers to both native forms of somatotropins and to analogs of native somatotropins provided that the analogs have sufficient protein identity with their parent compounds to have bioactivity as either a growth promoter or as a stimulant for milk production.

The phrase "animal somatotropin" refers to somatotropins originating from animals and includes somatotropins derived from either natural sources, e.g., pituitary gland tissue or from microorganisms transformed by recombinant DNA techniques to produce a naturally-occurring form of somatotropin. When, for example, a specific mammalian source is named, such as a bovine somatotropin, the somatotropin includes those derived from either natural sources or from transformed microorganisms.

The term "microorganism" as used herein refers to both single cellular prokaryotic and eukaryotic organisms such as bacteria, yeast, actinomycetes and single cells from higher plants and animals grown in cell culture. Transgenic animals are also known to those skilled in the art for production of heterologous polypeptides.

-6-

The term "native" refers to naturally-occurring forms of somatotropins which may have been derived from either natural sources, e.g., pituitary gland tissue or from microorganisms transformed by recombinant DNA techniques to produce a naturally-occurring  
5 form of somatotropin.

The term "vector" includes both cloning plasmids and plasmids for directing the expression of a somatotropin by virtue of the DNA encoding the somatotropin being operatively linked to a promoter capable of functioning in a microorganism. In some circumstances the  
10 term vector can also include chromosomal insertion of the DNA encoding the heterologous polypeptide.

Somatotropins are very similar in amino acid sequence and physical structure. Although the processes described in the Examples are directed toward bSt, the processes are equally applicable to any  
15 animal somatotropin, particularly other ~~mammalian~~ somatotropins, having the requisite cysteine residues available for conversion to serine.

During the oxidation step in the isolation of rbSt, reducible protein dimer and oligomer are formed by intermolecular disulfide  
20 bridges. To overcome this problem, rbSt analogs have been constructed. These analogs include those having the DNA sequence corresponding to the codons for cysteine at positions 181 and 189 changed to the codon for serine, two other analogs having the codon at either 181 or 189 changed to a serine codon, and one encoding a  
25 truncated rbSt protein which contains a translational stop codon at position 179. These four analogs were made as gene fusions and were expressed using expression vector pCFM414 and the E. coli strain AM343c. The vectors have been designated pRA-bSt/hyb-Ser181/9, pRA-bSt/hyb-Ser181, pRA-bSt/hyb-Ser189, and pRA-bSt/hyb-179T. A vector  
30 containing the DNA which encodes an unmodified rbSt product, pRA-bSt/hyb, was also constructed to serve as a control. All five of these vectors were found to express rbSt at high levels in the AM343c strain.

The higher relative potency of the cys → ser bSt is readily  
35 determined using hypophysectomized rats (Evans, H.M. and Long J.A., Anat. Rec. 21:61, 1921). Relative increases in total body weight are recorded using pituitary bSt, rbSt and various fractions of bSt analogs.



-7-

Administration of cys → ser bSt into dairy cattle is done according to known methods using any route effective to deliver the required dosage to the animal's circulatory system. Modes of administration include oral, intramuscular injections, subcutaneous  
5 injections and the use of timed-release implants. The preferred mode of administration is by subcutaneous injection using a timed-release implant. Appropriate vehicles for injections include physiologically compatible buffers such as sodium bicarbonate, sodium phosphate, or ammonium phosphate solutions. Timed-release implants are known in  
10 the art, e.g., U. S. Pat. 4,333,919.

The effective dosage range is from 1.0 to 200 milligrams per animal per day. The greater the amount of bSt given, the greater the resulting increase in growth, lactation or numbers of mammary parenchymal cells. Most preferably, the dosage range is from 5 to 50  
15 milligrams per day.

Because growth hormones are very similar in their amino acid sequences, hormones originating from one species can usually enhance the growth of other unrelated animal species. The bSt analogs of the present invention can be used to produce increased growth in the  
20 species in which native bSt has been shown to have growth-related bioactivity such as bovines, sheep, rats, salmon and chickens. The preferred animals are bovines used for beef such as bulls, heifers or steers.

Beef cattle are slaughtered just prior to reaching full maturity  
25 and size. Cys → ser bSt can be used to produce increased growth rates in beef cattle by administration any time between weaning until slaughter. Cys → ser bSt is administered to beef cattle for a minimum of 30 days and for a maximum of 450 days depending upon desired time of slaughter. Animals used for veal are typically  
30 slaughtered at approximately 6 months of age and 10 to 30 mg/day of cys → ser bSt is administered up until the age of slaughter to effectuate desired increases in growth rate.

For purposes of increasing lactation in bovines, particularly dairy cows, cys → ser bSt is administered between 30 and 90 days  
35 post-partum and continued for up to 300 days. Cys → ser bSt will also increase lactation in other commercial milk-producing animals such as goats and sheep.

Strains: The MC1000 (F<sup>-</sup>, araD<sub>139</sub>, del(araABC-leu)<sub>7679</sub>, galU,

-8-

trp<sub>am</sub>, malB<sub>am</sub>, rspL, relA, thi) (available in the Experiments with Gene Fusion Strain Kit, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York ) and JM83 (ara, del(lac-pro), rspL, phi80 lacZ delM15) (available from American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852) strains are used as competent cells for vector construction. The AM343c (F<sup>-</sup>, sup<sup>o</sup>, tonA) strain is used for inductions. It was derived from expression strain AM343C by curing the strain of the endogenous expression plasmid pCFM414-bGH. Strain AM343C (also designated UC<sup>®</sup>9801) was deposited at Agricultural/Research Service Culture Collection, Northern Regional Research Center, 1815 North University Street, Peoria, Illinois 61604, on 24 January 1989, and was given accession number NRRL B-18443. An alternative strain for expression is BST-1C (see PCT/US 88/00328, which is incorporated herein by reference).

Plasmids: The pUC9 (J. Vieira and J. Messing, Gene 19, pp. 259-268 (1982), available from Bethesda Research Laboratories) and pCFM414-bGH vectors were used in the constructions describe herein. The pCFM414-bGH vector (deposited with strain AM343C, above) was constructed from pCFM414 (U.S. Patent 4,710,473) by inserting an EcoRI/BamHI fragment that contains the trp promoter, and the trpL ribosome binding site from E. coli, and a synthetic bSt gene. The pTrp-BStml and the pTrp-BStmlb vectors are described in PCT/US 88/00328. Both of these vectors were constructed using a bSt cDNA gene (PCT/US88/00328, incorporated herein by reference). Both vectors have been modified in the DNA sequence encoding the bSt carboxyl-terminus. The pTrp-BStml vector has a BamHI restriction site immediately after the translational stop codon for bSt and contains a serine codon at position 189. The pTrp-BStmlb vector is similar to pTrp-BStml except that it contains a cysteine codon corresponding to position 189 of bSt.

Media: Media have been previously described (J. H. Miller, Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York).

DNA Preparation and Cloning protocols: Most DNA preperation and general techniques are done by techniques well known to those skilled in the art (see, T. Maniatis, et al, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1982) which is incorporated herein by reference). Specifically, DNA

-9-

is prepared as previously described (J.E. Mott, et al, EMBO J., 4, pp. 1887-1891 (1985)). Restriction digestions and ligations are performed according to the manufacturers' recommendations. Restriction enzymes and ligase are purchased from New England Biolabs. DNA fragments are isolated from agarose gels by one of two techniques. For fragments smaller than 800 bps, the bands are cut from the gel, crushed and frozen on dry ice in the presence of phenol. The aqueous phase is separated by centrifugation and ethanol precipitated. For fragments greater than 800 bp the band is cut from the gel and electro-eluted (T. Maniatis, et al, supra).

Plasmid Transformations: Cells to be transformed are grown overnight and subcultured 1/100. These cells are grown to mid-log phase, 5 ml is centrifuged and resuspended in 10 ml of cold 50 mM  $\text{CaCl}_2$ , and incubated on ice or at 4° C for at least one hour. The cells are again centrifuged and taken up in 0.5 ml 50 mM  $\text{CaCl}_2$ . Approximately 100  $\mu\text{l}$  of cells is incubated on ice with plasmid DNA for one hour. Cells are heat shocked for 7 min. at 37° C, and are plated on LB plates containing 100  $\mu\text{g}/\text{ml}$  ampicillin. Selections for runaway vectors are done at 27° C. Selections for non-runaway vectors are done at 37° C.

Oligomer Purification: Oligomers are synthesized and purified as previously described (PCT/US88/00328).

Formation of Synthetic Fragments: The synthetic DNA fragments for the introduction of the termination codon at position 179 are formed by heating oligonucleotides JM-1 and JM-2 (Chart 2) in the presence of ligation buffer to 100° C and allowing the mixture to cool to room temperature. To generate the four oligonucleotide block for the 181 and 189 serine substitutions, oligomers JM-4 and 5 are first kinased with high specificity  $\text{p}^{32}$ -labeled gamma-ATP and then hybridized to JM-3 and JM-6 (Chart 3). The four oligonucleotide structure is ligated and isolated from a non-denaturing polyacrylamide gel.

Induction Protocol: Cells containing vectors derived from the pCFM414 runaway vector are grown overnight at 27° C and subcultured 1/50 in broth supplemented with 100  $\mu\text{g}/\text{ml}$  ampicillin. The cells are grown in a New Brunswick air shaker at approximately 250 rpm and at 27° C. When the cells reach an O.D. 550 of between 0.2 and 0.4, a sample for SDS PAGE is taken and the cultures are transferred to a

-10-

37° C air shaker. Cells containing vectors derived from pURA-1 are induced as previously described (PCT/US88/00328).

SDS PAGE: The SDS PAGE protocol has been previously described (J.E. Mott, et al, Proc. Natl. Acad. Sci. USA, 82, pp. 88-92 (1985)).

5       The sequencing kit Sequenase<sup>TM</sup> is purchased from United States Biochemical. Sequencing is performed according to the manufacturers' recommendations. DNA for double-stranded sequencing is prepared by the method of L. B. Agellon and T. T. Chen, Gene Anal. Techn. 3 pp.86-89 (1986). For sequencing double-stranded DNA, the annealing  
10       protocol is modified as follows: 3 $\mu$ l of 2M NaOH, 2mM EDTA is added to 12 $\mu$ l double-stranded plasmid DNA and incubated for 5 minutes at room temperature, 3 $\mu$ l of 100ng/ $\mu$ l primer is added and the mix is incubated for 10 minutes followed by the addition of 6 $\mu$ l of 3M sodium acetate. After an additional 5 minute incubation, 100 $\mu$ l of ice-cold 95%  
15       ethanol is added and the DNA is precipitated for 20 minutes on dry ice. The DNA is pelleted, rinsed with 70% ethanol and vacuum dried. After resuspension in 8 $\mu$ l water and 2 $\mu$ l sequencing buffer, the sequencing is continued according to the manufacturer's directions. 2.5 $\mu$ l of the dGTP termination mixes A, C, G and T are pipetted into  
20       individual wells of a microtiter plate and pre-warmed at 37°C. The Sequenase enzyme is diluted 1:8 in ice cold TE buffer. For the labeling reaction, 1 $\mu$ l of 0.1M DTT, 2 $\mu$ l of the diluted label mix, 2 $\mu$ l of <sup>35</sup>S-dATP and 2 $\mu$ l of diluted Sequenase are added to the annealed DNA and the reactions incubated for 2 minutes at room temperature.  
25       For the termination reaction, 3.5 $\mu$ l of the labeling reaction is added to each termination well (A, C, G and T) and incubated for an additional 5 minutes. The reactions are stopped with 4 $\mu$ l of stop mix. After boiling the reactions for 2 minutes at 75°C, 4 $\mu$ l of each reaction are loaded onto a sequencing gel.

30       The temperature sensitive runaway vector pCFM414-bGH expresses high levels of rbSt. The vector contains a synthetic rbSt gene which is expressed from the trp promoter and uses the trpL ribosomal binding site for initiation of translation. This synthetic rbSt gene and the cDNA bSt gene (PCT/US88/00328) both have a unique PstI  
35       restriction site at the sequence which corresponds to amino acids 90 and 91. Thus hybrid bSt genes can be made using this common in-frame site. A hybrid gene, bSt/hyb, containing the sequence encoding the N-terminal region of the synthetic gene and the carboxyl terminus of

-11-

the cDNA gene is expressed to produce rbSt protein at high levels when cloned into the pCFM414 vector. Using the bSt/hyb gene the distal portion of the bSt gene can be altered using the unique restriction sites located in the cDNA segment. The cDNA, for example, contains a MstII restriction site at the sequences which encode amino acids 175, 176 and 177. These can be used to replace the sequence for the carboxyl terminus with synthetic DNA fragments which alter the cysteine codon at positions 181 and 189 or prematurely terminate translation. In order to take advantage of restriction sites, the hybrid genes must be placed in a vector in which these sites remain unique.

Example 1 Construction of a bSt Gene Encoding a Truncated bSt Lacking the Small Loop

1. Construction of pDH23

The pUC-9 vector (J. Vieira and J. Messing, Gene, 19, pp.259-268 (1982), available from Bethesda Research Laboratories, Gaithersburg, Maryland 20877) is a constitutive high copy number vector derived from pBR322. It contains the gene for ampicillin resistance and the lac promoter expressing the lacZ' beta-galactosidase gene. The lacZ' gene encodes a small N-terminal peptide fragment which is capable of functionally complementing a peptide fragment produced by the lacZdelM15 beta-galactosidase gene (Vieira and Messing, supra). This complementation can be visualized by using the chromogenic substrate X-Gal (Bethesda Research Laboratories, Gaithersburg, MD) which turns blue in cells with functioning beta-galactosidase. pUC9 has been modified to contain unique restriction sites within the sequence for lacZ' which are referred to as the polyclonal sites. DNA fragments which are inserted into these sites destroy the ability of the lacZ' peptide to be produced and to complement the lacZdelM15 beta-galactosidase protein. Such cells do not turn blue in the presence of the X-Gal indicator.

The pUC-9 vector does not contain a MstII restriction site but does contain unique sites in lacZ' polylinker for EcoRI, BamHI, PstI, and HindIII. The pUC-9 vector is modified to eliminate the PstI and the HindIII sites which would interfere with the hybrid gene construction and subsequent modification. This is accomplished by digesting the vector with HindIII and HinII. A HinII site is also present in the polyclonal site. The HindIII site is filled with PolA

-12-

klenow, the vector is ligated and transformed into competent JM83 cells. Resultant plasmids are screened and a candidate selected which has lost the HindIII and PstI restriction sites but retained the BamHI and EcoRI sites. This alteration to the polyclonal site  
5 does not affect the ability of the lacZ' peptide to complement since the lacZ' gene retains the proper reading frame. The resultant vector is designated pDH23. The unique EcoRI and BamHI restriction sites permit the cloning of the synthetic bSt gene.

2. Construction of bSt/hyb a Synthetic bSt - bSt cDNA Hybrid  
10 Gene

The synthetic bSt gene is isolated from plasmid pCFM414-bGH as an EcoRI/BamHI fragment and is cloned into the pDH23 vector which is previously digested with BamHI and EcoRI. The EcoRI/BamHI fragment also contains the trp promoter and the trpL ribosome binding site.  
15 The resultant vector is designated pDH-bSt. In this vector the PstI and BamHI restriction sites in the bSt gene are unique.

A PstI/BamHI fragment is isolated from the pTrp-BStml vector. This vector contains the bSt cDNA which is expressed from the trp promoter and uses the trpL ribosomal binding site. The bSt gene in  
20 pTrp-BStml is modified to encode a serine at position 189. The pTrp-BStml vector is digested with PstI and BamHI and a fragment of about 315 bp is isolated which contains the DNA sequence for the carboxyl terminus of bSt. This fragment is ligated into plasmid pDH-bSt, which is previously digested with PstI and BamHI replacing the PstI  
25 to BamHI sequence of the synthetic bSt gene. The resultant vector from this cloning is designated pDH-bSt/hyb-189S. This construction contains the unique restriction site MstII which is located between the PstI and BamHI restriction sites of the bSt gene.

3. pDH-bSt/hyb-189S Vector Derivatives

30 Three vectors are derived from pDH-bSt/hyb-189S of section 2. For the first cloning, the pDH-bSt/hyb-189S vector is digested with PstII and BamHI and the large vector fragment is isolated. A PstI/BamHI fragment is isolated from pTrp-BStmlb (PCT/US88/00328). pTrp-BStmlb is similar to pTrp-BStml except that it contains a syn-  
35 thetically derived oligonucleotide fragment cloned between the MstII and BamHI restriction sites. This fragment is identical to the fragment described in Chart 3 except that it contains the cysteine codon TGC at the positions corresponding to amino acids 181 and 189.

-13-

The codon usage is optimized and a HindIII restriction site is engineered into the sequence between codons 181 and 189. The PstI/BamHI fragment is isolated from pTrp-BStmlb and ligated into the large fragment of the pDH-bSt/hyb-189S vector to generate pDH-bSt/hyb. The bSt gene of pDH-bSt/hyb encodes the natural bSt.

For the next two clonings the pDH-bSt/hyb-189S is digested with MstII and BamHI and the large vector fragment from the digestions is isolated. A synthetic fragment derived from four oligonucleotides is shown in Chart 3. This synthetic fragment is designed to be inserted into the MstII/BamHI restriction sites. The insertion of this fragment generates a gene encoding a bSt protein with serine at amino acid positions 181 and 189. This fragment is ligated to the large MstII/BamHI fragment of pDH-bSt/hyb-189S to create the second vector pDH-bSt/hyb-Ser181/9. In this vector the restriction sites PstI, MstII, HindIII, and BamHI are unique.

The third vector derived from pDH-bSt/hyb-189S is made by inserting the synthetic fragment shown in Chart 2. This fragment encodes a translational stop codon corresponding to position 179 of the bSt. The fragment is ligated to the MstII/BamHI fragment of pDH-bSt/hyb-189S to generate a bSt gene that encodes a truncated bSt lacking the small loop. This vector is called pDH-bSt/hyb-179T.

The pDH-bSt/hyb-Ser181/9 and pDH-bSt/hyb-179T constructions are confirmed by DNA sequence analysis.

Example 2 Construction of bSt Genes with Individual Serine Substitutions Encoded at either Position 181 or 189

This example sets forth the cloning strategy for constructing genes with individual serine codons at position 181 or 189. Two starting vectors are used, pDH-bSt/hyb and pDH-bSt/hyb-Ser181/9 (Example 1). pDH-bSt/hyb encodes the natural bSt gene and pDH-bSt/hyb-Ser181/9 has serine codons for amino acids 181 and 189. Both vectors have a unique HindIII restriction site located between the sequence for these two codons. Digestion of these vectors with EcoRI and HindIII generates two fragments from each vector. The 850 bp fragment contains the trp promoter and about 95% of the bSt gene inclusive of the 181 codon. The 850 bp fragments are exchanged between the starting vectors in the cloning so that the fragment from pDH-bSt/hyb containing a cysteine codon at 181 is introduced into the pDH-bSt/hyb-Ser181/9 vector which has the serine codon at 189, and

-14-

conversely the 850 bp fragment containing the serine 181 codon from pDH-bSt/hyb-Ser181/9 is cloned into the pDH-bSt/hyb vector containing the 189 cysteine codon. The results of this cloning are two vectors, pDH-bSt/hyb-Ser181 and pDH-bSt/hyb-Ser189, which express bSt analogs containing single cysteine to serine substitutions at either amino acid position 181 or 189.

#### Example 3 pCFM414 Clonings

For high level expression the modified genes are cloned into the expression vector pCFM414-bGH. pCFM414-bGH is digested with EcoRI and BamHI and the large vector fragment is purified, thus removing the endogenous bSt gene. The vectors pDH-bSt/hyb, pDH-bSt/hyb-Ser181/9, pDH-bSt/hyb-Ser181, pDH-bSt/hyb-Ser189 and pDH-bSt/hyb-179T are digested with EcoRI and BamHI, and an 870 bp fragment containing the trp promoter, trpL ribosome binding site and the entire bSt gene is isolated from each. The fragments are individually ligated to the pCFM414 vector fragment and transformed into competent cells of E. coli strain MC1000. Plasmid DNA for the individual constructions is prepared. The resultant vectors are designated pRA-bSt/hyb, pRA-bSt/hyb-Ser181/9, pRA-bSt/hyb-Ser181, pRA-bSt/hyb-Ser189 and pRA-bSt/hyb-179T.

#### Example 4 Expression of the pRA Vectors

The vectors pRA-bSt/hyb, pRA-bSt/hyb-Ser181/9, pRA-bSt/hyb-Ser181, pRA-bSt/hyb-Ser189 and pRA-bSt/hyb-179T are transformed into competent cells of E. coli AM343c. The cultures are induced as described above, and a six hour post-induction sample from each culture is analyzed by SDS PAGE. All constructs show high level expression of their respective rbSt proteins. Alternatively, E. coli strain BST-1C is used for expression (PCT/US88/00328).

#### Example 5 Construction of an Alternative Expression System for the bSt Analog Containing Serine at Positions 181 and 189

Vector pDH-bSt/hyb-Ser181/9 as described above contains between its EcoRI and PstI sites the trp promoter, trpL ribosome binding site, and the synthetic bSt DNA sequence encoding amino acids 1 to 90 of bSt, from the PstI site to the MstII site it contains a sequence derived from the cDNA gene corresponding to amino acid positions 91 to 176 (Example 1), and from the MstII to BamHI restriction sites contains sequences derived from the synthetic oligonucleotide block



-15-

(Example 1). The portion of the gene derived from the synthetic bSt gene can be deleted by digesting pDH-bSt/hyb-Ser181/9 with EcoRI and PstI and isolating the large vector fragment. The deleted segment can be replaced with an EcoRI, PstI restriction fragment from pTrp-BStm4 (PCT/US88/00328), which contains the trp promoter, and the trpL ribosome binding site and the DNA sequence from the cDNA encoding amino acids 1 to 90 of bSt. This DNA also contains a GCC to GCT codon change at the codon corresponding to the second alanine of bSt which results in improved expression (PCT/US88/0328). The resultant vector is called pDH-bStm4-Ser181/9.

To introduce the bStm4-Ser181/9 cDNA gene into the pURA expression vector (PCT/US88/00328), both the pDH-bStm4-Ser181/9 and pURA-1 vectors are digested with EcoRI and BamHI restriction enzymes. The large vector fragment from pURA-1 and the smaller bSt gene fragment from pDH-bStm4-Ser181/9 are isolated and ligated together with a BamHI transcriptional termination fragment of rpoC (PCT/US88/00328). The ligated DNA is used to transform competent cells of MC1000. The resultant vector, pURA-bStm4-Ser181/9 is identical to pURA-4 (PCT/US88/00328) except that it contains the 181 and 189 cys → ser modifications.

For expression, the vector is transformed into the BST-1C strain (PCT/US88/00328) and induced as previously described (PCT/US88/00328).

Example 6 Construction of bSt Genes with Serine Replacing Cysteine at Position 53 or 164

Ser-53 or ser-164 is made from DNA encoding bSt having serine at those positions by oligonucleotide-directed mutagenesis on a double-stranded DNA plasmid, using the following procedure:

The oligonucleotide primer is phosphorylated by incubating 4 μl of oligonucleotide (4 μg) with 54 μl of water, 7 μl of buffer (0.5 M Tris-HCl, pH 7.4, 0.1 M MgCl<sub>2</sub>, 0.1 M dithiothreitol), 2 μl of 50 mM ATP, and 3 μl of 10 units/μl T4 polynucleotide kinase to a final volume of 70 μl at 37° C for 60 minutes. The reaction mixture is extracted with phenol and ether, evaporated to dryness, and dissolved in 3 μl of H<sub>2</sub>O. Two μl of this phosphorylated oligonucleotide is mixed with 15 μl of denatured plasmid DNA (denatured by incubating 2 μg of DNA in 12 μl with 3 μl of 2 N NaOH and 2 mM EDTA for 15-20 minutes at room temperature), followed by the addition of 6 μl of 3 M

-16-

sodium acetate, and 100  $\mu$ l of ethanol. The mixture is kept in dry ice for 15-30 minutes and the precipitate is collected by centrifugation, washed with 70% ethanol, dried, and dissolved in 25  $\mu$ l of 10 mM Tris-HCl, pH 7.4 and 1 mM EDTA. This solution is added with 1  $\mu$ l of the phosphorylated oligonucleotide, 20  $\mu$ l of water, 7.5  $\mu$ l of 1 M Tris-HCl, pH 8.3, 10  $\mu$ l of 0.2 M NaCl, 25  $\mu$ l of 0.1 M  $MgCl_2$ , 20  $\mu$ l of 10 mM dNTP, 17.5  $\mu$ l of 5 mM ATP, 20  $\mu$ l of 0.4 M  $\beta$ -mercaptoethanol, 16  $\mu$ l of Klenow enzyme (80 units), 20  $\mu$ l of T4 DNA ligase (8,000 units), and 18  $\mu$ l of T4 gene 32 protein (32  $\mu$ g). The reaction mixture, in a total volume of 200  $\mu$ l, is incubated at 37° C for 1 hour, mixed with NaCl to final 0.2 M, extracted with phenol and ether, and precipitated with ethanol. The precipitate is dissolved in 100  $\mu$ l of 10 mM Tris-HCl, pH 7.4 and 1 mM EDTA and used for transformation of *E. coli* DH1, MC1000 or other appropriate strains. Depending on the transformation efficiency, 2-20  $\mu$ l of the mutagenized plasmid are used for transformation.

The transformants carrying the plasmid with the mutation are screened for by colony hybridization, using the mutagenesis oligonucleotide as the probe and selecting washing conditions to detect the mutation over the wild-type background. The procedures for hybridization are as described in T. Maniatis, E.F. Fritsch and J. Sambrook, Molecular Cloning. In general, 1-2  $\mu$ g of the oligonucleotide probe is phosphorylated with 100-200 MCi of ( $\gamma$ - $^{32}P$ )ATP and 10-20 units of T4 polynucleotide kinase in 50 mM Tris-HCl, pH 7.8, 10 mM  $MgCl_2$ , and 10 mM dithiothreitol, in a total volume of 20  $\mu$ l. Hybridization is carried out in 5x Denhardt's, 5x SSC and 0.1% SDS at 42° C. The filters are washed in 5x SSC and 0.1% SDS at a temperature which allows the oligonucleotide probe to remain annealed to the changed sequence and dissociated from the wild-type sequence. Transformants that hybridize strongly with the oligonucleotide probe are selected and DNA sequencing is carried out to confirm the altered sequence.

Using the above techniques, oligonucleotide-directed mutagenesis to generate DNA encoding bSt with serine at position 53 or 164 is carried out with plasmid pURA-m4 (PCT/US88/00328). To change cys-53 to serine, an oligonucleotide with the sequence GTTGCCCTTCTCTTCTCTGA-AAC is used for mutagenesis and as a probe for colony hybridization with washing temperature for the filters at 51° C. The plasmid with the change is called pURA-m4-53ser. The serine substitution at

-17-

position 164 is generated with the oligonucleotide CTGCTCTCCTCTTTCCGGAAGG using 51° C for washing of filters and the resulting plasmid is called pURA-m4-164ser.

Example 7 Construction of a bSt Gene with Serine Replacing  
5 Cysteine at Both Positions 53 and 164.

This strategy uses the PstI site located at codon 90 of the bSt gene to splice together the bSt sequences with single serine substitutions at position 53 or 164. To remove other PstI sites in the vector for convenient manipulation, the bSt sequence with serine at position 164 is subcloned into pDH23 by isolating the EcoRI/BamHI small fragment containing the trp promoter and the bSt gene from pURA-m4-164ser and ligating it to the EcoRI/BamHI large vector fragment isolated from pDH23. The resulting plasmid pDH-m4-164ser has a single PstI site at codon 90 of the bSt gene. The EcoRI/PstI large vector fragment containing the 3' half of the bSt sequence with serine at position 164 is isolated from pDH-m4-164. This fragment is ligated to the small EcoRI/PstI fragment containing the trp promoter and the 5' half of the bSt encoding sequence with serine at position 53 isolated from pURA-m4-53ser. The resulting plasmid is named pDH-m4-53/164ser.

For high level expression of bSt with serine at positions 53 and 164, the EcoRI/HindIII small fragment containing the trp promoter and the bSt sequence is isolated from pDH23-m4-53/164ser. This fragment is ligated to the EcoRI/HindIII large vector fragment isolated from pURA-m4. The resulting plasmid is named pURA-m4-53/164ser.

**Example 8**      **Construction of a bSt Gene with Serine at Positions 54, 164, 181, and 189.**

To construct a vector encoding bSt with serine replacing cysteine at positions 53, 164, 181, and 189, the EcoRI/MstII small  
30 fragment carrying the trp promoter and most of the bSt coding sequence with serine at positions 53 and 164 is isolated from pDH-m4-53/164ser. This fragment is ligated to the EcoRI/MstII large vector fragment containing the 3' bSt coding sequence with serine at positions 181 and 189 isolated from pDH-bSt/hyb-ser181/9. The  
35 resulting plasmid is named pDH-m4-53/164/181/189ser. For high level expression of rbSt with all 4 cysteine residues replaced by serine, the EcoRI/BamHI small fragment containing the trp promoter and the bSt coding sequence with serine at positions 53, 164, 181 and 189 is

-18-

isolated from pDH-m4-53/164/181/189ser (Fragment I). The EcoRI/BamHI large vector fragment (Fragment II) and the 350 bp BamHI fragment carrying the transcription terminator for the *E. coli* genes rpoBC (Fragment III) are isolated from pURA-m4. Fragments I, II and III are ligated and a plasmid with the terminator in the correct orientation is selected and named pURA-m4-53/164/181/189ser.

Example 9 Expression of bSt with Serine at One or More of the Positions 53, 164, 181 and 189.

The vector pURA-m4-53ser, pURA-m4-164ser, pURA-m4-53/164ser or pURA-m4-53/164/181/189ser is transformed into *E. coli* BSt-1C and induced as described in patent application PCT/US88/00328.

Example 10 Biological Activity of Cys → Ser bSt Analogs

AM343c cells transformed with pRA-bSt/hyb-Ser181/9 were grown in 200 L of beer using a modified Luria Broth. Initially the cells were grown at 27°C, pH 7 until an A<sub>550</sub> of 1 was obtained. Induction was caused by a temperature shift to 37°, a pH shift to 6 and the addition of yeast extract (PCT/US 88/00328). rbSt is harvested from the cells collected by centrifugation as follows:

1) Cells are first lysed with lysozyme and the lysate is then washed with Tergitol.

2) The DNA is sheared and the inclusion bodies are washed and then solubilized with sodium lauroyl sarcosine.

3) rbSt so produced is oxidized and folded by stirring with air for 24 hrs.

4) Sodium lauroyl sarcosine is removed by treatment with Dowex-1x4.

5) rbSt mixture is chromatographed over DEAE-Sepharose and the rbSt fractions are recovered and lyophilized.

For the rat bioassay, hypophysectomized, female, Sprague-Dawley rats are purchased from Charles River Laboratories, Inc. The rats are divided into test groups of 7 rats per group. A standard pituitary-derived bSt for control is dissolved in 0.15 M NaCl/0.03 M sodium bicarbonate buffer, pH 10.8. The pH of the solution is adjusted to 9.5 and the solution is diluted to a protein concentration of 2 mg/ml with a similar buffer at pH 9.5. The test rbSts are prepared as lyophilized powders containing Tween 80 and mannitol in sodium bicarbonate buffer. These are reconstituted to a 10 mg/ml solution which is then diluted 1:5 to make stock solutions. In

-19-

addition to the cys → ser analogs, a preparation of recombinantly produced natural rbSt is used as a control. Each animal in the test groups is injected twice daily (100 µl/injection) for a total dosage of 7.5, 15, 30 or 60 mg protein/day for a total of nine days. The rats are weighed daily throughout the treatment period, except weekends.

For the cow bioassay, lactating Holstein cows were used. These cows were free of metabolic disorders, mastitis, disease and medication before assignment to the study. The cows were blocked in four replicates of five cows each based on the average milk yield on days 3 and 2 before the first injection of test compound. Cows between 122 and 289 days postpartum were used for the study. The five cows of each block were assigned randomly to one of three experimental groups: 5 mg and 15 mg each daily of cys → ser rbSt, rbSt process control and non-injected control group. Cows were injected at about 0745 to 0815 hours daily; i.e., 2 to 3 hours after milking. The same lyophilized preparations used for the rat study were used to make 10 mg/ml stock solutions for the injections. Injections were made once daily in the semitendinous muscle and continued for 7 days. Cows were milked at 0500 and 1600 hours according to the routine milking schedule and methodology of the dairy. Calibrated milk jars were used to estimate individual cow milk weights at each milking for each cow in the study from 3 to 5 days after the 7 days of injections, i.e., a total of 15 days. Milk yield was adjusted to 3.5% fat corrected milk.

A summary of the analytical profiles of the cys → ser analog preparation and the control preparation of recombinantly-produced natural rbSt is given in Table 1.

-20-

Table 1

| Assay              | control           | cys → ser *       |
|--------------------|-------------------|-------------------|
| RIA                | 92%               | 18%               |
| SDS-PAGE (Monomer) | 97.8%             | 98.0%             |
| 5 IEF (Major pI)   | 8.1 and 8.2       | 8.1 and 8.2       |
| Endotoxins         | 1.5 EU/mg protein | 6.2 EU/mg protein |
| HPLC-SEC           | Polymer 0.00%     | Polymer 0.00%     |
|                    | Monomer 97.97%    | Monomer 98.99%    |
|                    | 84.34% by wt.     | 90.93% by wt.     |
| 10 RP-HPLC         | Ox. 116.7%        | Ox. 120.32        |
|                    | Red. 0.73%        | Red. 0.2%         |
| DNA Content        | 0.15 ng/mg        | 0.115ng/mg        |
| E. coli Protein    | <0.05%            | 0.052%            |

15 \*This analog had both cysteines at positions 181 and 189 replaced with serine (181, 189).

Generally the two preparations are similar in their analytical profiles. However, in the radioimmune assay the cys → ser bSt analog was only 18% of the standard as compared to 92% for the control.

20 Milk yields were also higher for cows treated with the cys → ser bSt (181, 189) analog as compared to the natural rbSt control (Table 2).

Table 2

Fat Corrected Milk Yield

25 (kg milk/day)

| Compound     | Dose (mg) |          |           |
|--------------|-----------|----------|-----------|
|              | <u>0</u>  | <u>5</u> | <u>15</u> |
| Control      | 24.6      |          |           |
| rbSt Control |           | 25.0     | 25.7      |
| 30 Cys → ser |           | 27.6     | 26.2      |
| (181, 189)   |           |          |           |

35 When injected into hypohysectomized rats, the cys → ser (181, 189) rbSt is more active than the control preparation in inducing body weight gain (Table 3).

-21-

Table 3

Body Weight Gain Potency in  
Hypophysectomized Rats

|    | <u>Compound</u> | <u>Relative to Pituitary</u>  |               | <u>Relative to rbSt Control</u> |               |
|----|-----------------|---|---------------|---------------------------------|---------------|
|    |                 | Potency   |               | Potency                         |               |
|    |                 | <u>Estimate</u>   | <u>95% CL</u> | <u>Estimate</u>                 | <u>95% CL</u> |
| 5  | Pituitary bSt   | 1.0   |               |                                 |               |
|    | rbSt Control    | 1.42  | 1.09-1.87     | 1.0                             |               |
|    | Cys → ser       | 1.78  | 1.36-2.35     | 1.25                            | 0.96-1.64     |
| 10 | Example 11      | Expression of bSt with Serine at Positions 181 and 189 using Vector pURA-bStm4-Ser181/189 |               |                                 |               |

Cells (BST-1C) were transformed with pURA-bStm4-Ser181/189 and grown in 200 liters of beer as previously described (U.S. patent application 157,275, filed 17 February 1988). The rbSt was harvested from the collected cells as in Example 10 except that it was necessary to repeat Steps 4 (sodium lauroyl sarcosine removal by Dowex-1x4 treatment) and 5 (chromatography on DEAE-Sepharose). This was done for two preparations from the fermentation. In vitro analytical data are given in Table 4.

Table 4

Analyses of rbSt Analog Preparations

|    | <u>Assay</u>        | <u>Preparation A</u> |  | <u>Preparation B</u> |  |
|----|---------------------|----------------------|--|----------------------|--|
|    |                     |                      |  |                      |  |
|    | RIA                 | 41%                  |  | 31%                  |  |
|    | HPLC                |                      |  |                      |  |
| 25 | Oxidized (Wt %)     | 72.68                |  | 74.05                |  |
|    | Reduced (Wt %)      | 0.78                 |  | 0.21                 |  |
|    | SEC                 |                      |  |                      |  |
|    | Polymer (Area %)    | 0.92                 |  | 0.00                 |  |
|    | Dimer (Area %)      | 4.05                 |  | 3.95                 |  |
| 30 | Monomer (Area %)    | 93.51                |  | 94.15                |  |
|    | Monomer (Wt%)       | 84.17                |  | 79.55                |  |
|    | Total Protein by    |                      |  |                      |  |
|    | Amino Acid Analysis | 98.0%                |  | 95.3%                |  |

-22-

## CHART 1. Amino Acid Sequence Of Bovine Somatotropin

1  
ala phe pro ala met ser leu ser gly leu phe ala asn ala val  
5  
20  
leu arg ala gln his leu his gln leu ala ala asp thr phe lys  
40  
10 glu phe glu arg thr tyr ile pro glu gly gln arg tyr ser ile  
60  
gln asn thr gln val ala phe cys phe ser glu thr ile pro ala  
15  
pro thr gly lys asn glu ala gln gln lys ser asp leu glu leu  
80  
20 leu arg ile ser leu leu leu ile gln ser trp leu gly pro leu  
100  
25 gln phe leu ser arg val phe thr asn ser leu val phe gly thr  
120  
30 ser asp arg val tyr glu lys leu lys asp leu glu glu gly ile  
leu ala leu met arg glu leu glu asp gly thr pro arg ala gly  
35  
140  
gln ile leu lys gln thr tyr asp lys phe asp thr asn met arg  
160  
40 ser asp asp ala leu leu lys asn tyr gly leu leu ser cys phe  
180  
45 arg lys asp leu his lys thr glu thr tyr leu arg val met lys  
190  
cys arg arg phe gly glu ala ser cys ala phe  
50



-23-

CHART 2. Assembly of a Synthetic DNA Fragment for Introduction of a Translational Terminator at Codon 179.

A. Sequence of Individual Oligomers

5 JM-1 3' GGATAATGTGGGAGT  
JM-2 3' CCCACATTATCCCTAG

B. Sequence of the Synthetic Fragment

5' TGAGGGTGTAAATAGG  
10 CCCACATTATCCCTAG

CHART 3. Construction of a Synthetic Fragment for Cysteine to Serine Codon Changes at Positions 181 and 189.

15

1) Sequence of the Individual Oligomers used to Construct the Synthetic Fragment.

JM-3 3' CTTTGCTGCCCTAAAGTATTGGGAGT  
JM-4 3' GGATAATCTTTCGCCTCCTTCGAAGTGG  
20 JM-5 3' CCCAATACTTTAGGGCAGCAAAGCCACTT  
JM-6 3' CGAAGGAGGCGAAAGATTATCCCTAG

2) Configuration of the Oligomers in the Synthetic Fragment

5' \_\_\_\_\_ JM-3 \_\_\_\_\_ JM-4 \_\_\_\_\_ 3'  
25 3' \_\_\_\_\_ JM-5 \_\_\_\_\_ JM-6 \_\_\_\_\_ 5'

3) Sequence of the dsDNA Synthetic Fragment

5' -TGAGGGTTATGAAATCCCGTCGTTTCGGTGAAGCTTCCTCCGCTTTCTAATAGG  
CCCAATACTTTAGGGCAGCAAAGCCACTTCGAAGGAGGCGAAAGATTATCCCTAG-5'  
30

4) Location of Key Restriction Sites

MstII HindIII BamHI  
\_\_\_\_\_ 1815 \_\_\_\_\_ 1895 \_\_\_\_\_

-24-

## CLAIMS

1. Mammalian somatotropin in which at least one of the cysteine residues corresponding to residues 54, 164, 181 and 189 is replaced with serine.  
5
2. A mammalian somatotropin according to claim 1 in which both cysteine residues corresponding to residues 181 and 189 are replaced with serine.
- 10 3. A mammalian somatotropin according to claim 1, in which the cysteine residue corresponding to residue 181 is replaced with serine.
- 15 4. A mammalian somatotropin according to claim 1, in which the cysteine residue corresponding to residue 189 is replaced with serine.
- 20 5. A mammalian somatotropin according to claim 1, which is bovine somatotropin.
6. A mammalian somatotropin according to claim 2, which is bovine somatotropin.
- 25 7. A mammalian somatotropin according to claim 3, which is bovine somatotropin.
8. A mammalian somatotropin according to claim 4, which is bovine somatotropin.
- 30 9. A mammalian somatotropin according to claim 1, wherein at least one cysteine residue at 53 or 164 is replaced with serine.
- 35 10. A mammalian somatotropin according to claim 9, wherein the cysteine residue 53 is replaced with serine.
11. A mammalian somatotropin according to claim 9, wherein the cysteine residue 164 is replaced with serine.

-25-

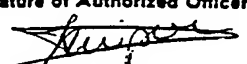
12. A mammalian somatotropin according to claim 9, wherein the cysteine residues at 53 and 164 are replaced with serine.
13. A mammalian somatotropin according to claim 1, wherein the cysteine residues at 53, 164, 181 and 189 are replaced with serine.
14. A mammalian somatotropin according to claim 9, which is bovine somatotropin.
15. A mammalian somatotropin according to claim 10, which is bovine somatotropin.
16. A mammalian somatotropin according to claim 11, which is bovine somatotropin.
17. A mammalian somatotropin according to claim 12, which is bovine somatotropin.
18. A mammalian somatotropin according to claim 13, which is bovine somatotropin.
19. A method for enhancing the growth of animals that comprises treatment of the animals with an effective amount of a composition of claim 1.
20. The method of claim 19 wherein the animal is a species of bovine.
21. A method for increasing milk production in a female ruminant comprising the administration of an effective amount of a composition of claim 1.
22. The method of claim 21 wherein the ruminant is a dairy cow.
23. A vector comprising DNA coding for a mammalian somatotropin having at least one of the cysteine residues corresponding to residues 54, 164, 181 and 189 changed to serine.

-26-

24. The vector of claim 23 which is capable of directing the expression of the somatotropin-like protein.
25. A microorganism hosting a vector comprising DNA coding for a mammalian somatotropin-like protein having at least one of the cysteine residues corresponding to residues 54, 164, 181 and 189 of the closest related native somatotropins changed to serine.
26. The microorganism of claim 25 selected from the bacterial genus, *Escherichia*.

# INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/05445

|  |  |   |
|--|--|---|
| <b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply indicate all) *<br>According to International Patent Classification (IPC) or to both National Classification and IPC<br>IPC <sup>5</sup> : C 12 N 15/18, C 12 P 21/02, C 07 K 13/00, A 61 K 37/36,<br>A 23 K 1/165, C 12 N 1/21, //(C 12 N 1/21, C 12 R 1:19)  |  |   |
| <b>II. FIELDS SEARCHED</b>   |  |   |
| Minimum Documentation Searched <sup>7</sup>  |  |   |
| Classification System  | Classification Symbols   |   |
| IPC <sup>5</sup>   | C 12 N, C 12 P, C 07 K, A 61 K, A 23 K   |   |
| Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched *  |  |   |
|  |  |   |
| <b>III. DOCUMENTS CONSIDERED TO BE RELEVANT *</b>  |  |   |
| Category *   | Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>   | Relevant to Claim No. <sup>13</sup>   |
| Y  | EP, A, 0109748 (CETUS CORP.)<br>30 May 1984<br>see the whole document<br>(cited in the application)<br><br>--  | 1-26  |
| Y  | Eur. J. Biochem., vol. 153, no. 3, 1985,<br>FEBS,<br>T. TOKUNAGA et al.: "Synthesis and<br>expression of a human growth hormone<br>(somatotropin) gene mutated to change<br>cysteine-165 to alanine",<br>pages 445-449,<br>see the whole article<br>(cited in the application)<br><br>-- | 1-26  |
| A  | Chemical Abstracts, vol. 110, no. 11,<br>13 March 1989, (Columbus, Ohio, US),<br>page 212, abstract no. 89909p,<br>& JP, A, 62255500 (KYOWA HAKKO KOGYO<br>CO., LTD), 7 November 1987  |   |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&amp;" document member of the same patent family</p> </div> </div> |  |   |
| <b>IV. CERTIFICATION</b>   |  |   |
| Date of the Actual Completion of the International Search  |  | Date of Mailing of this International Search Report   |
| 16th March 1990  |  | 23 APR 1990   |
| International Searching Authority  |  | Signature of Authorized Officer   |
| EUROPEAN PATENT OFFICE   |  | <br>Mme N. KUIPER |

**ANNEX TO THE INTERNATIONAL SEARCH REPORT  
ON INTERNATIONAL PATENT APPLICATION NO.**

US 8905445  
SA 32912

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 11/04/90. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s) | Publication<br>date |
|---|---------------------|----------------------------|---------------------|
| EP-A- 0109748                             | 30-05-84            | AU-B- 563962               | 30-07-87            |
|   |                     | AU-A- 2008683              | 03-05-84            |
|   |                     | BE-A- 898016               | 15-02-84            |
|   |                     | CH-A- 669394               | 15-03-89            |
|   |                     | CH-A- 669395               | 15-03-89            |
|   |                     | DE-A- 3376271              | 19-05-88            |
|   |                     | EP-A- 0192811              | 03-09-86            |
|   |                     | EP-A, B 0218825            | 22-04-87            |
|   |                     | EP-A- 0234599              | 02-09-87            |
|   |                     | FR-A, B 2534594            | 20-04-84            |
|   |                     | GB-A, B 2130219            | 31-05-84            |
|   |                     | JP-A- 1020096              | 24-01-89            |
|   |                     | JP-A- 61028392             | 08-02-86            |
|   |                     | JP-A- 61028393             | 08-02-86            |
|   |                     | JP-A- 61028384             | 08-02-86            |
|   |                     | JP-A- 61028394             | 08-02-86            |
|   |                     | JP-A- 59093093             | 29-05-84            |
|   |                     | US-A- 4588585              | 13-05-86            |
|   |                     | US-A- 4853332              | 01-08-89            |
|   |                     | US-A- 4737462              | 12-04-88            |
|   |                     | US-A- 4518584              | 21-05-85            |
| -----                                     |                     |                            |                     |